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Antifungal Treasure House: Effects of Bioactive Secondary Metabolites from *Trichoderma* strains against Fungal Phytopathogens

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ABSTRACT
Chemical fungicides and unsustainable methods used to control plant diseases necessitate a shift from traditional management practices. The genus Trichoderma, known for its diverse biological activities, has attracted researchers' attention in understanding the roles of its bioactive secondary metabolites. This study investigates the effects of volatile organic compounds (VOCs) and solvent extracts from Trichoderma strains (Trichoderma viride, Trichoderma harzianum, Trichoderma ressei, Trichoderma longibrachiatum, and Trichoderma asperellum) against phytopathogens Aspergillus niger, Macrophomina phaseolina, and Fusarium oxysporum. Dual culture assays, conducted through direct and indirect confrontations, demonstrated the antagonistic nature of Trichoderma strains. T. viride and T. harzianum inhibited the growth of M. phaseolina by 82.09% and 84.53%, respectively, while T. asperellum, T. harzianum, and T. longibrachiatum reduced F. oxysporum growth by 92.1%, 80%, and 74.43%, respectively. In the second method, T. viride inhibited M. phaseolina growth by 71.7%, and T. harzianum inhibited F. oxysporum growth by 73.33%. The solvent extract of T. viride exhibited a 21.67mm inhibition zone against F. oxysporum, and T. harzianum showed a 20.33mm zone against A. niger. These in-vitro findings suggest that the bioactive secondary metabolites produced by T. viride and T. harzianum in plate cultures and liquid extracts possess antifungal potential against phytopathogens. Utilizing these bioactive compounds offers a sustainable and non-chemical approach to managing issues caused by phytopathogens in plants.
Keywords – Antifungal; *Trichoderma;* Bioactive secondary metabolite

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INTRODUCTION

Plant diseases are claimed to be a key factor in the world's annual level of food production, which varies depending on the source and is estimated to be between 10 to 40%. Plant diseases actively contribute to the deterioration of natural resources in farmland. The five most significant global crops—rice, wheat, maize, potatoes, and soybeans are the ones that are most frequently impacted by plant mycoses, in accordance with the Food and Agriculture Organization of the United Nations (FAO). In total, over 19,000 species of fungi that contribute to crop illnesses are currently recognised on a global scale [19]. Due to an over use of chemical fertilisers aimed at improving agricultural productivity and yield, there is chronic ecological damage [16]. The general health of terrestrial and aquatic ecosystems as well as non-target animals like beneficial insects, particularly pollinators, are all negatively impacted by application of synthetic pesticides [19]. Integrated Pest Management (IPM), organic farming, and various other sustainable approaches to agriculture are used to safeguard the ecosystem from the harmful effects of synthetic chemicals [19]. Plant pathologists and industrial corporations have recently expressed a great deal of enthusiasm in using biological control agents [14]. Biopesticides and biofertilizers, based on natural ingredients, living microbes, or their metabolites can be used as a viable alternative to conventional

pesticides to maintain good yields with no adverse environmental effects [16], [19]. *Trichoderma* spp. have drawn a lot of attention as potential agents for the biological control of plant pathogenic fungi, and they seem like viable candidates for subsequent successful commercialization [14]. Utilizing antagonistic soil microbes is one method of biological control. Anamorphic fungi that have been isolated mostly from soil and decaying organic matter belong to the genus *Trichoderma* [15]. *Trichoderma* fungi have received a great deal of research attention and are employed as bioactive components in biopesticides and biofertilizers [12]. These fungi constitute the most susceptible microbes to both natural and man-made substances and toxins, and they can efficiently digest some of them, such as hydrocarbons, chlorophenolic compounds, polysaccharides, and xenobiotic pesticides. They can also utilise a variety of nutrition sources, are considered the most resistant microbes to toxins [21]. Numerous soil phytopathogens, including fungi like *Fusarium oxysporum*, *Rhizoctonia solani*, *Sclerotium rolfsii*, and *Verticillium dahliae*, are antagonistically affected by *Trichoderma* species [10]. *Trichoderma* strains generate both volatile and non-volatile secondary metabolites, several of which inhibit biological organisms they are not directly in contact with. Such deterrents are regarded as antibiotics [13]. These compounds, including 6-n-pentyl-6H-pyran-2-one (6PP), gliotoxin, viridin, harzianopyridone, harziandione, gliotoxin, viridin, and peptaibols, are bioactive in nature and are produced as secondary metabolites depending on the strain [20]. The two primary categories of known antibiotic-active secondary metabolites are (i) low molecular weight and volatile metabolites/ non-polar metabolites and (ii) high molecular weight and polar metabolites/ non-volatile metabolites. Simple aromatic molecules, volatile terpenes, isocyanide, and nonpolar chemicals with high vapour pressure are examples of low molecular weight and volatile metabolites. The volatile organic compounds (VOCs) disperse over a long distance through networks within the soil environment, improving the performance of the organism by altering the physiology of rival species. Peptaibols and other polar, high-molecular-weight metabolites may exert their effect directly through interactions between *Trichoderma* species and their antagonists [11]. Phytopathogens such as *Macrophomina phaseolina*, *Fusarium oxysporum*, and *Aspergillus niger* are a cause of serious threats to plants. In ware onions, black mould (*Aspergillus niger*) is frequently found [3] and still *A. niger*'s infection on onions has not been controlled completely. Throughout India, a large number of agricultural crops, particularly vegetables, are heavily afflicted another famous pathogenic fungi *Fusarium oxysporum*. These fungi cause the *Fusarium* yellows or wilts disease, which affects many vegetables [9]. Chickpea (*Cicer arietinum*) *Fusarium* wilt was brought on by strain *Fusarium oxysporum* [6]. It is the main pathogen contributing to tomato vascular wilt [18]. Another primary cause of the charcoal rot disease, which considerably reduces the output of a range of oilseed crops, is still *Macrophomina phaseolina* (Tassi) Goid [4]. Even sunflower is frequently observed with a *M. phaseolina* infection [17]. In this investigation, we examined the antagonistic potential and secondary metabolite production of strains of *Trichoderma viride* M2497373, *Trichoderma longibrachiatum* M2497368, *Trichoderma harzianum* M2497371, *Trichoderma ressei* M2497364, *T. asperellum,* and *T. longibrachiatum* against three phytopathogens that adversely affect farming crops: *Macrophomina phaseolina*, *Fusarium oxysporum*, and *Aspergillus niger*.

MATERIAL AND METHODS

Fungal strains

The fungal strains used in this study were obtained from the Research Laboratory of the Department of Microbiology and Biotechnology, School of Sciences, Gujarat University. The following six strains of *Trichoderma* fungi (*T. viride* M2497373, *T. longibrachiatum* M2497368, *T. harzianum* M2497371, *T. ressei* M2497364, *T. asperellum* KT582304, and *T. longibrachiatum*) were maintained on potato dextrose agar. The phytopathogenic strains Macrophomina phaseolina, *Fusarium oxysporum*, and *Aspergillus niger* were also included (PDA, HiMedia). Under sterile conditions, these fungal strains were cryopreserved using 30% glycerol. Before any experiment, the cultures were thawed and cultured in potato dextrose broth at 26 °C for 48-72 hours, and then inoculated onto potato dextrose agar plates.

In vitro Dual culture plate assay

Mycelial blocks of the phytopathogen and *Trichoderma*, approximately 5-7mm in size, were excised from the edges of 3-5 day-old culture plates. These blocks were then positioned opposite each other on the margins of petri plates containing PDA media. The control dishes contained only the mycelial blocks of the pathogenic fungi. The plates were incubated at 26°C with a 12-hour light/dark cycle for 7 days [18]. A slight modification was made to assess the presence of volatile organic compounds (VOCs). The media was divided into two sections to establish indirect contact between the antagonists and the test fungi. The following formula [13] was used to calculate the percentage inhibition of radial growth of the pathogenic fungi *In vitro*.

$$I = \{(C-T) \times 100\} \div C$$

Where, I = Percentage of mycelial growth of inhibition C = Mycelial growth in control. T = Mycelial growth in treated [9].

The identical procedure was employed for both direct and indirect confrontation in the dual culture plate assay, with the same steps repeated every other day of incubation until reaching a total of 7 days.

Liquid fermentation and extraction of Secondary Metabolites

A 5-7mm mycelial block obtained from each of the six *Trichoderma* strains, after five days of growth, was inoculated into 100 ml of pre-treated potato dextrose broth medium (PDB) in 250 ml Erlenmeyer flasks. The fermentation process took place on an orbital shaker for a duration of 14 days [8]. Afterward, the culture filtrate was collected as a supernatant by centrifugation, while the fungal biomass formed a pellet. The culture filtrate and fungal biomass of each *Trichoderma* strain were utilized for the extraction process. To extract the secondary metabolites, ethyl acetate was added to the culture filtrate in an equal ratio (1:1 v/v), and a biphasic extraction was performed. This extraction process, involving liquid-liquid partition, was repeated three times, vigorously shaking to facilitate the formation of two distinct layers (solvent and culture filtrate) [18]. The extraction from the culture biomass was carried out using the same procedure. The excess solvent was then evaporated from the extracts using a rotary evaporator operating at 45°C and 100RPM to obtain concentrated extracts.

***In vitro* antifungal activity of fungal bioactive-extracts**

The three fungal pathogens' spore suspensions were surfaced on PDA plates using a sterile spreader, and three wells were bored in the PDA plates using a cork borer. Each well in the PDA plates were injected by 50ul of the bioactive extracts, following which they were incubated at 25°C. As a control, ethyl acetate was used in one of the wells. The diameter of the zone of inhibition was measured following a 72-hour incubation period [11].

RESULTS AND DISCUSSION

It is observed from this experiment that all the tested bioagents produced volatile antifungal metabolites having a significant effect in reducing the mycelial growth. In direct confrontation method of Dual culture plate, it was recorded and calculated each alternate days till 7th day, *Trichoderma viride* M2497373 (MJ1) Table 1. inhibited radial growth of *Macrophomina phaseolina* (F1) by 82.09%, *Fusarium Oxysporum* (F2) by 65.55% and, *Aspergillus niger* (F3) by 65.72%. *Trichoderma longibrachiatum* M2497368 (MJ2) Table 2. inhibited growth of F1 by 66.68%, F2 by 57.8% and, F3 by 47.22%. *Trichoderma harzianum* M2497371 (MJ3) Table 3. inhibited growth of F1 by 84.53%, *F. Oxysporum* by 80% and, *Aspergillus niger* by 62.05%. *T. ressei* M2497364 (MJ4) Table 4. inhibited growth of F1 by 59.34%, F2 by 66.66% and, F3 by 55.55%. *Trichoderma longibrachiatum* (MJ5) Table 5. inhibited growth of F1 by 65.02%, F2 by 74.43% and, F3 by 46.27%. *Trichoderma asperellum* KT582304 (MJ6) Table 6. inhibited growth of F1 by 56.69%, F2 by 92.1% and F3 by 44.11%. In in-direct confrontation method of Dual culture plate, the media was partitioned in two halves and no direct contact was there within *Trichoderma* strains and the pathogenic fungi. MJ1 inhibited radial growth of F1 by 71.7%, F2 by 65.53% and, F3 by 53.69%. MJ2 inhibited growth of F1 by 66.68%, F2 by 57.8% and, F3 by 47.22%. MJ3 inhibited growth of F1 by 66.65%, F2 by 73.33% and, F3 by 52.77%. MJ4 inhibited growth of F1 by 37.41%, F2 by 56.66% and, F3 by 52.77%. MJ5 inhibited growth of F1 by 49.58%, F2 by 40% and, F3 by 31.47%. MJ6 inhibited growth of F1 by 26.08%, F2 by 63.15% and, F3 by 44.11%. Graph 1. and Graph 2. represents in-vitro antagonistic activity in Percentage reduction of radial growth in pathogenic fungi by *Trichoderma* strains through Direct as well as indirect confrontation method (nVOCs check) in Dual Culture plate assay. The resultant observations and percentages obtained from in-vitro dual culture plate assay, it is clear as *Trichoderma* strains are in direct contact, the % reduction was visible more than the one in which the media was partitioned. In agar well diffusion, assay Table 7. and Table 8., Ethyl acetate extract of MJ1 filtrate was observed to give 8.67mm zone of inhibition towards F1, 21.67mm zone of inhibition towards F2, 10.33mm Zone of inhibition against F3, and that of crude biomass was not determined. Ethyl acetate extract of MJ2 filtrate was observed to give 13.33mm zone of inhibition towards F1, 12.33mm zone of inhibition towards F2, 10.66mm Zone of inhibition against F3 and that of crude biomass was 11mm, 12mm and 10.66mm against respective pathogenic strains. The solvent extract of MJ3 filtrate was observed to give 13.33mm, 12mm and 20.33mm zone of inhibition towards F1, F2, and F3, whereas solvent extract of crude biomass was 9mm, 10.33mm and 14mm against respective pathogenic strains. The solvent extract of MJ4 filtrate was observed to give 7.66mm, 10.33mm and 13.33mm zone of inhibition towards F1, F2, and F3, whereas solvent extract of crude biomass gave inhibition zone of 5mm towards F1, 6.33mm towards F2 and towards F3 it was not determined against respective pathogenic strains. The solvent extract of MJ5 filtrate was observed to give 15mm, 14.38mm and 13.66mm zone of inhibition towards F1, F2, and F3, whereas solvent extract of crude biomass was 9.66mm, 5.66mm and 9.33mm towards respective pathogenic strains. Ethyl acetate extract of MJ6 filtrate was observed to give

14.67mm zone of inhibition towards F1, 13mm zone of inhibition towards F2, 14.67mm Zone of inhibition against F3, and that of crude biomass gave inhibition zone of 8.33mm against F1, 8.33mm towards F2, and 8mm towards F3. Ethyl acetate gave no detrimental antifungal activities towards any of the pathogenic strains used. Based on results of Dual culture plate assay and Agar well-diffusion assay, it indicates that the volatile organic compound with good antifungal abilities are observed to be present in *Trichoderma harzianum* M2497371 (MJ3) *and Trichoderma viride* M2497373 (MJ1) (Figure 1.). Other strains of *Trichoderma* represent moderate and low antifungal activity towards pathogenic fungi. In previous literature also it has been recorded that *Trichoderma harzianum* strains as well as *Trichoderma viride* strains has shown some serious impression against pathogenic fungi. For example*, T. virde* was responsible for 66.66% reduction in radial growth in *F. oxysporum*, 88.8% reduction in *Colletotrichum capsici* [9], 64% reduction in *M. phaseolina* [5]. *T. harzianum* inhibited radial growth of *Alternaria porri* by 79.9% [1]. *T. harzianum* isolates has been studied; *Trichoderma* isolates retarded the radial mycelial growth of *F. moniliforme* by 32.5% and 45%. *T. harzianum* had a good inhibitory impact on *B. sorokiniana* when applied to PDA (45.71%) [2]. The isolates of *P. theae* and *F. solani* were antagonistic to *T. viride* strain SDRLIN1 to the extent of 50.51% and 63%, respectively. *Trichoderma harzianum* strains CMML20–26 and CMML20–27 were reducing growth of *Fusarium oxysporum* CMML21–2 by 81.14% and 72.05% [8]. According to study, antifungal activity of T. harzianum extract (50ul) towards strains such as *Aspergillus fumigatus, Aspergillus clavatus*, *Aspergillus terreus*, *Fusarium semitectum, Fusarium graminiarum*, and *Cladosporium* was recorded with zone of inhibition- 22mm, 21mm, 37mm,19mm, 16mm, and 19mm [7].

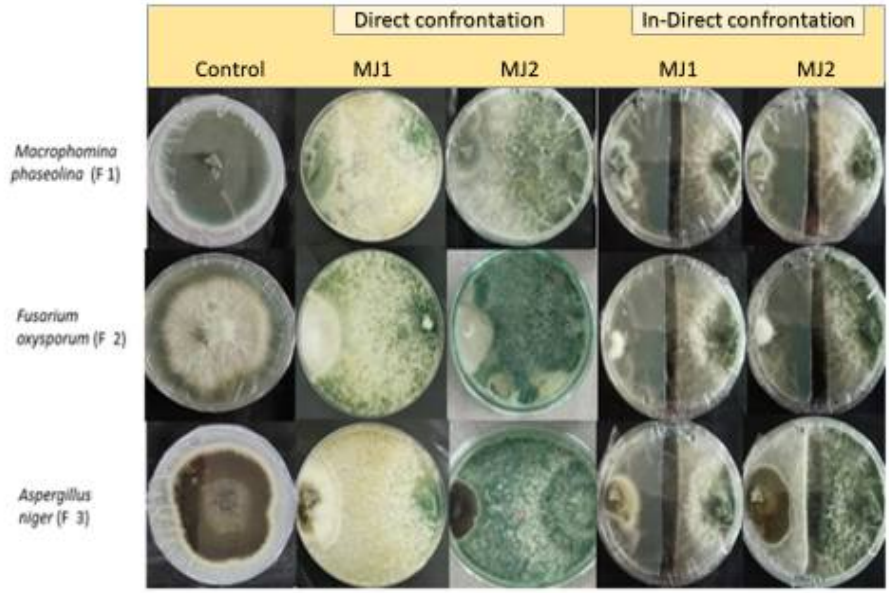


Figure 1. Antagonistic action of *Trichoderma viride* M2497373 and *Trichoderma harzianum* M2497371 against *M. phaseolina, F. oxysporum,* and *A. niger*

	MJ1					
Pathogen	non-VOCs			VOCs check		
	2nd day	4th day	6th day	2nd day	4th day	6th day
F1	24.05	56.66	**82.09**	31.44	31.13	**71.7**
F2	8.3	39.71	65.55	41.66	34.95	65.53
F3	13.3	34.5	65.72	41.65	27.35	53.69

Table 1. Percentage inhibition of pathogen's radial growth by Antagonistic bioactivity of *Trichoderma viride* M2497373 against pathogens

Pathogen	MJ2					
	non-VOCs			VOCs check		
	2nd day	4th day	6th day	2nd day	4th day	6th day
F1	13	61.13	66.68	27.7	43.33	56.09
F2	25	33.33	57.8	22.25	46.04	38.9
F3	36.7	46.2	47.22	40	19.07	56.5

Table 2. Percentage inhibition of pathogen's radial growth by Antagonistic bioactivity of *Trichoderma longibrachiatum* M2497368 against pathogens

	MJ3					
Pathogen	non-VOCs			VOCs check		
	2nd day	4th day	6th day	2nd day	4th day	6th day
F1	20.33	57.76	**84.53**	25.88	34.43	66.65
F2	16.67	46.04	**80**	22.16	42.85	**73.33**
F3	31.65	46.42	62.05	33.3	29.75	52.77

Table 3. Percentage inhibition of pathogen's radial growth by Antagonistic bioactivity of *Trichoderma harzianum* M2497371 against pathogens

	MJ4					
Pathogen	non-VOCs			VOCs check		
	2nd day	4th day	6th day	2nd day	4th day	6th day
F1	22.22	56.66	59.34	46.27	57.77	37.41
F2	13.91	57.14	66.66	5.58	52.38	56.66
F3	28.3	47.6	55.55	30	48.82	52.77

Table 4. Percentage inhibition of pathogen's radial growth by Antagonistic bioactivity of *Trichoderma ressei* M2497364 against pathogens

	MJ5					
Pathogen	non-VOCs			VOCs check		
	2nd day	4th day	6th day	2nd day	4th day	6th day
F1	33.33	63.33	65.02	25.94	53.33	49.58
F2	22.25	55.57	**74.43**	11.16	49.23	40
F3	28.3	45.21	46.27	35	48.82	31.47

Table 5. Percentage inhibition of pathogen's radial growth by Antagonistic bioactivity of *Trichoderma longibrachiatum* strain against pathogens

	MJ6					
Pathogens	non-VOCs			VOCs Check		
	2nd day	4th day	6th day	2nd day	4th day	6th day
F1	18.75	36.11	56.69	25	5.55	26.08
F2	33.33	65.38	**92.1**	25	42.3	63.15
F3	18.75	24	44.11	7.6	28	44.11

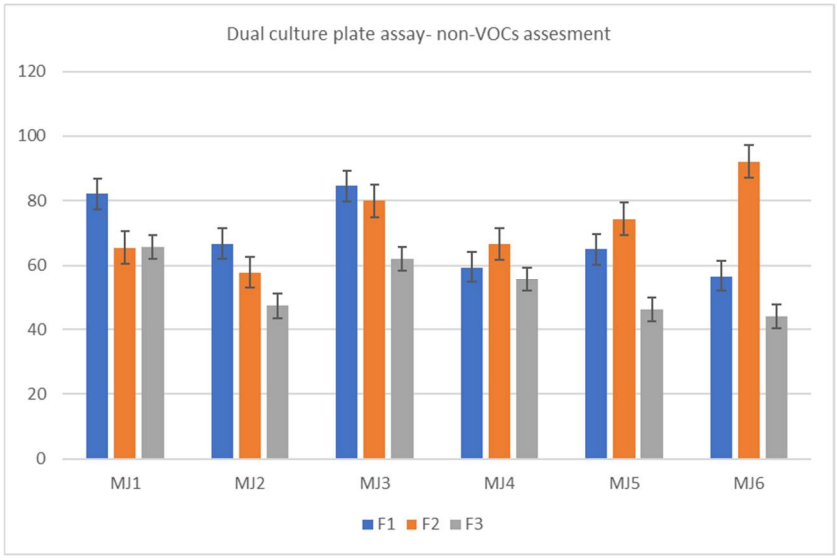
Table 6. Percentage inhibition of pathogen's radial growth by Antagonistic bioactivity of *Trichoderma asperellum strain* against pathogens

Pathogen	**Zone of inhibition(mm) of Solvent Extract from filtrate**						
	MJ1	**MJ2**	**MJ3**	**MJ4**	**MJ5**	**MJ6**	**control**
F1	8.67 ± 1.15	13.33 ± 1.24	16.33 ± 1.15	7.66 ± 0.57	15 ± 0	14.67 ± 1.15	N.D
F2	**21.67 ± 2.08**	12.33± 0.94	12 ± 1	10.33 ± 1.15	14.38 ± 0.57	13 ± 1	N.D
F3	10.33 ± 1.53	10.66 ± 0.94	**20.33 ± 2.52**	13.33 ± 1.52	13.66 ± 1.52	14.67 ± 1.52	N.D

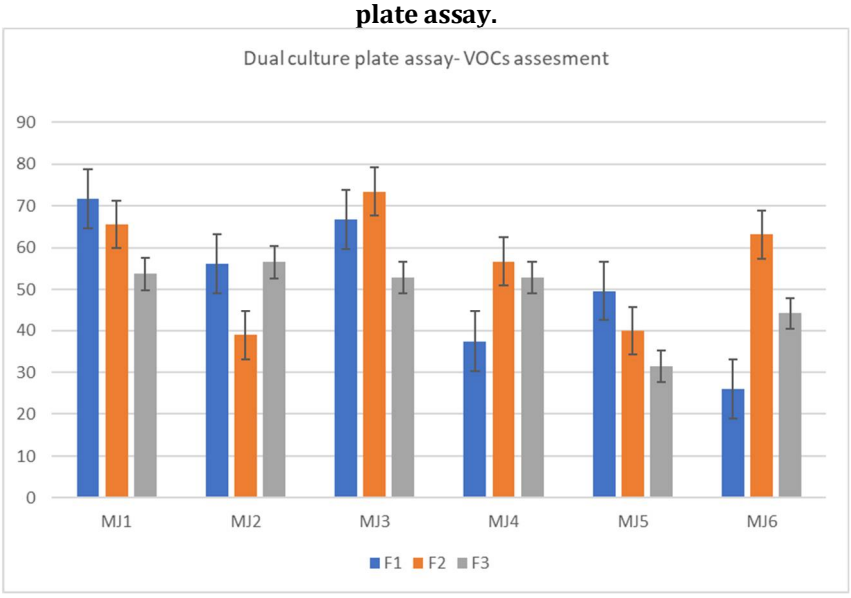
Table 7. Antifungal activity of solvent extracts of filtrate against fungal pathogens

Pathogen	***Zone of inhibition(mm) of Solvent Extract from crude biomass***						
	MJ1	***MJ2***	***MJ3***	***MJ4***	***MJ5***	***MJ6***	***control***
F1	*N.D*	*11 ± 0.81*	*9 ± 0*	*5 ± 1.732*	*9.66 ± 0.57*	*8.33 ± 1.52*	*N.D*
F2	*N.D*	*12 ± 0.81*	*10.33 ± 0*	*6.33 ± 0.57*	*5.66 ± 0.57*	*8.33 ± 1.53*	*N.D*
F3	*N.D*	*10.66 ± 1.24*	*14 ± 0*	*N.D*	*9.33 ± 1.15*	*8 ± 1*	*N.D*

Table 8. Antifungal activity of solvent extract of biomass against fungal pathogens



Graph 1. In-vitro Antagonistic activity in Percentage reduction of radial growth in pathogenic fungi by *Trichoderma* strains through Direct confrontation method (nVOCs check) in Dual Culture plate assay.



Graph 2. In-vitro Antagonistic activity in Percentage reduction of radial growth in pathogenic fungi by *Trichoderma* strains through indirect confrontation method (VOCs check) in Dual Culture plate assay.

CONCLUSION

In this study, *Trichoderma* strains were subjected to primary and secondary screening to assess their antifungal properties using the Dual Culture Plate assay and Agar Well diffusion assay. The findings indicate the presence of potent bioactive secondary metabolites in *Trichoderma* species that exhibit antifungal activity against plant pathogens. While all *Trichoderma* strains exhibited some level of antifungal activity, strains MJ2, MJ4, MJ5, and MJ6 showed moderate to low activity compared to MJ1 and MJ3. Interestingly, solvent extracts from the culture filtrate demonstrated better results than those from the crude biomass extracts.

Trichoderma viride M2497373 (MJ1) displayed exceptionally high antagonistic activity against *Macrophomina phaseolina* (F1) in both direct and indirect confrontations (see Table 1). Its solvent extract also exhibited significant antifungal activity against *Fusarium oxysporum* (F2) (see Table 7). *Trichoderma harzianum* M2497371 (MJ3) also exhibited good antagonistic activity against F1 and F2 (see Table 3), with its solvent extract displaying a substantial zone of inhibition, indicating high antifungal activity (see Table 7). These two strains, MJ1 and MJ3, hold promise for further exploration to enhance the production of

secondary metabolites with good yields, facilitating the characterization of the bioactive antifungal compounds present in these strains. These noteworthy results provide hope for future prospects in utilizing the extracted compounds/metabolic components to improve plant systems and employ them as applications against phytopathogenic fungi, thus combating fungal infections in plants.

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Competing Interests
The authors have declared that no competing interest exists

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